IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	J. Carman
FOR:	METHODS FOR PRODUCING APOMICTIC PLANTS
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Commissioner for Patents
Washington, D.C., 20231

Sir:

DECLARATION OF JOHN G. CARMAN UNDER 37 C.F.R. § 1.132

JOHN G. CARMAN hereby declares as follows:

1. That he is the inventor of the invention described and claimed in the above-identified U.S. Patent Application (hereinafter, "the application"), which is a continuation of U.S. Serial No. 09/018,875, filed February 5, 1998, which claims the benefit of U.S. Provisional Application No. 60/037,211, filed February 5, 1997.

2. That he received a B.S. from Brigham Young University in 1976, an M.S. in Botany and Range Science from Brigham Young University in 1978, and a Ph.D. in plant genetics from Texas A&M

University in 1982.

3. That after receiving his Ph.D. he was employed an Assistant Professor from 1982 to 1988, as an Associate Professor from 1988 to 1997, and as a Professor of Plant Genetics from 1997 to the present at Utah State University, Logan, Utah.

4. That he is the author or coauthor of more than 34 refereed journal articles, 9 chapters in books, and that he has delivered many invited presentations at national and international scientific meetings concerning his discoveries in the phylogeny, embryology, ecophysiology, developmental biology, and genetics of gametophytic apomicts.

5. That he has conducted experiments according to the methods for producing apomictic plants described and claimed in the application, which experiments are described and summarized as follows.

Materials and Methods

Plant Materials. *Sorghum*, *Tripsacum*, and *Antennaria* lines used in this study are listed in Tables 1, 2, and 3, respectively. These plants were grown in greenhouses, growth chambers, and field plots using standard procedures.

Phenology and Embryology. Photoperiodism requirements for flowering for all lines collected were compiled, and additional photoperiodism data were obtained from greenhouse and field observations by noting days to flowering from planting (*Sorghum*), days to flowering from the end of vernalization (*Antennaria*), and days to flowering following transfer from noninductive to inductive photoperiods (*Sorghum*, *Tripsacum* and *Antennaria*).

Pistils for embryological analyses were cleared and analyzed using differential interference contrast (DIC) microscopy (M.D. Peel et al., Meiotic Anomalies in Hybrids Between Wheat and Apomictic *Elymus rectisetus* (Nees in Lehm.) A. Löve & Connor, 37 Crop Sci. 717-723 (1997) (of record in the present application)). For *Sorghum* and *Tripsacum*, integument and ovule lengths were recorded at the dyad, tetrad, functional megaspore, and 1-nucleate, 2-nucleate, 8-nucleate, and mature embryo sac stages (Fig. 1) for all parent lines (Tables 1 and 3). For *Antennaria* , integument and ovule lengths were recorded at the dyad, the 2-nucleate, and mature embryo sac stages (Fig. 1) for all parent lines (Table 2). Surviving megaspores were classified as functional megaspores after they had initiated rapid enlargement; the functional megaspore stage was considered in this study to be the first stage of active embryo sac formation. Functional megaspores with one nucleus were classified as 1-nucleate embryo sacs if they contained one or more large vacuoles. Eight-nucleate embryo sacs were considered mature

embryo sacs if the egg apparatus was fully formed and the polar nuclei were fused.

For each *Sorghum*, *Tripsacum*, and *Antennaria* parent, inner integument lengths at the dyad stage (MS, for megasporogenesis) were expressed as percentages of the mean inner integument length at maturity. For *Sorghum* and *Tripsacum*, mature inner integument lengths for these calculations were obtained by computing the mean of the median and all larger values for the 8-nucleate embryo sac stage (enlarged 8-nucleate embryo sacs). Inner integument length values at the mature embryo sac stage for *Sorghum* and *Tripsacum* were not used, because they tended to be smaller than those obtained from enlarged 8-nucleate embryo sacs. Embryo sacs of *Sorghum* and *Tripsacum* expand laterally during the latter stages of their maturation, which causes the integuments to appear shorter (Fig. 1), i.e. they curve back toward their origin. For *Antennaria*, mature integument lengths (*Antennaria* has only one integument) were obtained by computing the mean of the median and all smaller integument length values obtained from the mature embryo sac stage.

For *Sorghum* and *Tripsacum*, the beginning of embryo sac formation was considered to occur during the latter half of the tetrad stage. Hence, mean inner integument length at embryo sac initiation (ESI) was considered to be the average value of the median inner integument length value at the tetrad stage combined

with all inner integument length values larger than the median at the tetrad stage. Integument length values at the tetrad stage were expressed as percentages of mean inner integument length at integument maturity.

For *Antennaria*, early embryo sac formation (EES) was used instead of ESI and was considered to occur during the beginning half of the 2-nucleate embryo sac stage. Hence, mean integument length at the EES stage was considered to be the average value of the median integument length value at the 2-nucleate embryo sac stage combined with all integument length values smaller than the median at the 2-nucleate embryo sac stage. Integument length values at the 2-nucleate embryo sac stage were expressed as percentages of mean integument length at integument maturity (early mature embryo sac stage for *Antennaria*).

Five traits were used to clarify reproductive differences between the parents of *Sorghum*, *Tripsacum*, and *Antennaria* hybrids produced according to the present invention. The first was photoperiodism (P). For *Sorghum*, lines were classified as being derived from either natural photoperiod insensitive (PI) lines or from natural photoperiod sensitive (PS) lines. *Sorghum* lines derived from "conversion programs" (lines converted from PS to PI) were considered to be of PS origin and are labeled herein as photoperiod insensitive converted (PIC). They are considered of PS origin because conversion programs generally alter only one of many

maturity loci (Y.R. Lin et al., Comparative Analysis of QTLs Affecting Plant Height and Maturity Across the Poaceae in Reference to an Interspecific Sorghum Population, 141 Genetics 391-411 (1995); W.L. Rooney & S. Aydin, Genetic Control of a Photoperiod-sensitive response in *Sorghum bicolor* (L.) Moench, 39 Crop Sci. 397-400 (1999); P.Q. Craufurd et al., Adaptation of Sorghum: Characterization of Genotypic Flowering Responses to Temperature and Photoperiod, 99 Theor. Appl. Genetics 900-911 (1999)). In most cases, dominance at the Ma_1 locus, which confers strong PS, is converted to homozygous recessiveness through introgression (hybridization and backcrossing resulting in near isogenic lines), which confers PI. Hence, alleles at many maturity loci of the original sensitive line are believed to be re-established through repeated backcrossing of the conversion process (Y.R. Lin et al., *supra*; W.L. Rooney & S. Aydin, *supra*), and some of these alleles may have pleiotropic effects on ovule development. *Tripsacum* species are photoperiod sensitive long day or short day plants, and minimum-days-to-flowering varies among accessions. *Antennaria* species are photoperiod sensitive long day plants with highly variable requirements for photoperiod length and minimum-days-to-flowering.

Differences in MS (dMS) and ESI (dESI for *Sorghum* and *Tripsacum*) or EES (dEES for *Antennaria*) constituted the second and third traits used to clarify reproductive differences between the

parents of hybrids. The data were arranged such that dMS values were always positive, i.e. the MS mean from the parent line that was first to achieved the dyad stage (relative to inner integument maturity) was subtracted from the MS mean of the parent line that achieved the dyad stage when its inner integument was more mature. The same order of subtraction was followed for dESI or dEES, i.e. the mean ESI or EES value of the parent line that achieved the dyad stage first was subtracted from the mean ESI or EES value of the parent line that achieved the dyad stage last. In this respect, negative dESI or dEES values indicate that the parent (of a given hybrid) first to achieved the dyad stage was (1) last to initiate embryo sac formation or reach the early embryo sac stage, compared with the other parent, and (2) had a longer tetrad duration (*Sorghum* or *Tripsacum*) or tetrad plus 1-nucleate embryo sac stage (*Antennaria*) than the other parent.

The difference between the smallest parental ESI or EES value and the largest parental MS value of a parental pair (ESI or EES and MS values may be from the same or different parents) was the fourth trait used to differentiate parent lines of hybrids. Negative ESI-MS or EES-MS values indicate that embryo sac formation is initiated or reaches the early stage in one parent before the dyad stage is reached in the other parent, e.g. see Fig. 2. When either ESI-MS or EES-MS values are negative, the ESI or EES (smallest ESI or EES value) and MS (largest MS value) values used

are always from different parents. Positive values of ESI-MS or EES-MS may involve ESI or EES and MS values from the same or different parents.

The difference in tetrad stage duration (dTET, *Sorghum* and *Tripsacum*) or duration of the tetrad plus 1-nucleate stage (dTET1, *Antennaria*) was the fifth trait used to clarify differences between the parents of each hybrid. The same order of subtraction was followed for dTET or dTET1 as was followed for dMS and dESI or dEES, i.e., the mean TET or TET1 value of the parent line that achieved the dyad stage first was subtracted from the mean TET or TET1 value of the parent line that achieved the dyad stage last. In this respect, a negative dTET or dTET1 value indicates that the parent (of a given hybrid) first to achieve the dyad stage remained in the tetrad stage longer than the other parent. In this respect, the tetrad stage represents a "waiting" period of varying duration prior to active embryo sac formation. In general, at least 10 embryological measurements were made at each stage (dyad, tetrad, etc.). Means were generated and compared by one-way analysis of variance (ANOVA). Differences between means were calculated using the Student-Newman-Keuls method (Sigma Stat 2.0, Jandel Corporation).

Apomixis and other reproductive irregularities in hybrids or parent lines were documented. Apomixis in hybrids was correlated with phenological and embryological data obtained from the parent

lines. Phenological and embryological divergence ratings among parent lines that produced apomicts were characterized. For *Antennaria*, phenograms derived from morphological, isozymic, ecological, and ITS sequence data were studied to further identify genetic and ecological variables responsible for apomixis evolving in this genus.

Hybridization and genetic verification. Standard hybridization procedures were used to make crosses within *Sorghum* and *Tripsacum*, and immature hybrid embryos were excised and grown aseptically on N6 or MS medium (J. Berthaud et al., *Tripsacum*: Diversity and Conservation, in S. Taba, Maize Genetic Resources (CIMMYT 1995). For *Antennaria*, pistillate plants were isolated by placing pollination bags over the entire capitulescence. Pollination was accomplished by rubbing receptive pistillate inflorescences together with staminate heads at anthesis. Unpollinated control capitulescences were used to verify absence of apomixis of parental clones. Using standard procedures, hybrid *Antennaria* seeds were aseptically germinated (without vernalization) on MS medium three weeks to three months following pollination.

In most cases, hybrids were readily recognized as intermediates between the pistillate and staminate parents. However, some of the hybrids produced among *Tripsacum* species, which are generally self incompatible, were produced by open

pollination in a USDA quarantine greenhouse. Since a limited number of pollen parents were available for hybridization, DNA tests were conducted to determine paternity and confirm maternity. This was accomplished by evaluating alleles found in the hybrids and comparing them with alleles found in the purported parents. Material/entries consisted of 14 *Tripsacum* lines that were screened with 18 informative microsatellite (simple sequence repeat, SSR) markers. The DNA for testing came from the individual plants (leaf samples) of each entry. Data were generated on an ABI Prism™ 377 DNA sequencer and were presented as alleles scored as estimated fragment sizes in base pairs compared to size standards or controls. Accuracy of the scoring is reportedly 0.67 base pairs with reproducibility from the same DNA source of 0.99 +/- 0.01. These analyses were conducted independently by Celera AgGen, Davis, CA.

Results

Sorghum. Twelve *Sorghum* lines were chosen that differ in geographic origin and in their floral responses to photoperiod (Table 1). Regardless of photoperiodism, all lines except 9-2 reached the dyad stage at about the same time relative to integument maturity (Figs. 3 and 4). The exception, line 9-2, is of the caudatum race (J.M.J. De Wet, Systematics and Evolution of *Sorghum* set *Sorghum* (Gramineae), 69 Am. J. Bot. 477-484 (1978)) and

is photoperiod sensitive. Neither of these traits is unique among the 12 accessions studied (Table 1). However, 9-2 is unique in that it flowers very quickly under short day conditions, and this trait, minimum-time-to-flowering (see P.Q. Craufurd et al., *supra*), may in some way contribute to its distinct delay in onset of meiosis. Of 8330 accessions tested by the USDA (http://www.ars-grin.gov/cgi-bin/npgs/html/obvalue.pl?69070), 9-2 reached anthesis in the shortest time (20 days). Accession 9-2 (http://www.ars-grin.gov/cgi-bin/npgs/html/acc_search.pl?accid=agira) was collected in 1954 from the Rainlands Research Station, Wad-Medani, Sudan, and donated to the USDA by O. J. Webster, USDA-ARS, Crops Research Division, University of Nebraska, Lincoln.

In contrast with the dyad stage (Fig. 4), much divergence among lines was observed for percentage integument maturity at the onset of embryo sac formation (Fig. 5). This variation was largely responsible for distinct differences among lines in tetrad duration (Fig. 6). In this respect, some lines, such as 1-1, 1-2, and 9-2, experienced only a brief tetrad stage before initiating embryo sac formation, while other lines, such as 2-1, 9-1, 6-1, and 4-1, experienced a long tetrad stage (Fig. 6).

Apomictic embryo sac formation was not observed in parent lines (Table 1) even though >60 pistils of each accession (>120 pistils for most accessions) were embryologically characterized during stages diagnostic for apomixis (functional megaspore through

2-nucleate embryo sac stage). In contrast, both aposporous and diplosporous embryo sacs were observed in hybrids produced among certain parent lines (Table 4; Fig. 7).

Apomictic embryo sac formation was nearly entirely restricted to hybrids heterozygous for (1) genes controlling photoperiodism (photoperiod sensitivity genes and minimum-time-to-flowering genes, see P.Q. Craufurd et al., *supra*), (2) genes controlling time of onset of megasporogenesis relative to gross ovule development, and (3) genes controlling time of onset of embryo sac formation relative to gross ovule development (Table 4).

Diplosporous embryo sac formation (Fig. 7) tended to occur in hybrids heterozygous for photoperiodism and for genes regulating onset of meiosis and embryo sac formation such that dMS was large and ESI-MS was small or negative (Table 5). In contrast, aposporous embryo sac formation (Fig. 7) tended to occur in hybrids heterozygous for photoperiodism and for genes regulating onset of meiosis and embryo sac formation such that dESI and dTET were large (Table 5).

By applying the methods of the presently claimed invention to *Sorghum* germplasm, apomictic *Sorghum* hybrids (17% of pistils containing apomictic embryo sacs, hybrid 15A, Table 4, Fig. 7) were produced from parent lines that do not produce apomictic embryo sacs.

Tripsacum. Photoperiod sensitivity and minimum-time-to-flowering are highly variable traits in and among *Tripsacum* species. In general, temperate zone *T. dactyloides* from the great plains of North America are long day plants that flower in the early summer. In contrast, tropical forms of *T. dactyloides* from Mesoamerica and Central and South America are short day plants that flower in the late fall. Most other *Tripsacum* species are short day tropical plants, and minimum-time-to-flowering under photoperiod appropriate conditions varies substantially among them, e.g. diploids of *T. pilosum* flower 20 to 30 days before diploids of *T. laxum* and *T. bravum*. Based on the results described above with *Sorghum*, diploid sexual *Tripsacum* divergent in photoperiod responses were predicted to be well suited for conversion to apomixis provided that they also possess sufficient temporal variability for megasporogenesis and embryo sac formation.

Diploid sexual *T. zopilotense* initiates meiosis and embryo sac formation, relative to pistil length, more quickly than diploid sexual *T. dactyloides dactyloides* (Table 6). Furthermore, diploid sexual *T. dactyloides meridionale* and *T. pilosum* initiate meiosis quickly but embryo sac formation appears to be delayed(compare Table 6 with Figs. 8-10).

Values of dMS and ESI-MS for the parental pairs *T. dactyloides dactyloides* (WW-2435) / *T. floridanum* (MIA 34719) and *T. dactyloides dactyloides* (WW 1253) / *T. floridanum* (MIA 34719) were

calculated from Figs. 8 and 9 and were essentially the same, approximately 14.5 and 3.0, respectively. Furthermore, flowering occurs in long days in WW-2435 and WW 1253 but in short days in MIA 34719 (the common parent). Thus, in hybrids produced from these parental pairs, embryo sac formation signals, which confer competence for embryo sac formation to ovular tissues, should weakly overlap (ESI-MS ≈ 3.0) with megasporogenesis signals, and it was predicted that this should cause infrequent diplospory. Higher frequencies of diplospory are predicted to occur when ESI-MS values are lower or negative (discussed above). The large dESI values (≈13.5) for these parental pairs suggest that aposporous embryo sac formation might also infrequently occur in hybrids produced there from. Three hybrids, 8xFCD, FAx8B and 3BxFAI, were produced from these parental pairs. Of the 31 pistils analyzed at appropriate stages (from the functional megaspore stage to the 2-nucleate embryo sac stage), five (16%) were developing diplosporously and one (3%) contained an enlarged aposporous initial (Fig. 11). Normal sexual development was observed in all appropriately-staged parental pistils (50 WW 2435 pistils, seven WW 1253 pistils, and 44 MIA 34719 pistils).

The highly-sexual North American diploid *T. dactyloides* and *T. floridanum*, which were used as parents in the hybrids just described, were considered ideal forage grass candidates for validating the presently claimed invention by conferring apomixis

to them. Near obligate to obligate apomixis in *Tripsacum* can be achieved by (1) identifying additional photoperiodism and embryological variability among the sexual *Tripsacum* diploids, (2) hybridizing the more divergent forms with each other or with the weakly apomictic products discussed above, (3) doubling the chromosomes of the resulting hybrids, which intensifies apomixis expression, and (4) optionally backcrossing or hybridizing with additional lines to produce sexually sterile but apomictically fertile triploids. These methods of inducing and intensifying apomixis expression may be applied to (1) naturally occurring facultative apomicts, (2) facultative apomicts that arise from sexual or weakly apomictic lines fortuitously in breeding programs (see the following example), or (3) facultative weakly apomictic lines that are derived intentionally through the methods of the present patent.

Naturally-occurring weakly apomictic diploids (probably dihaploids) of Mesoamerican origin were considered ideal candidates for validating the presently claimed invention by synthesizing strongly apomictic polyploids from sexual and/or weakly apomictic diploids.

Tripsacum bravum, *T. laxum* and *T. pilosum* form agamic complexes like *T. dactyloides*, and each occurs at the sexual diploid and apomictic polyploid levels. However, unlike the *T. dactyloides* agamic complex, diploids among these smaller agamic

complexes are rare (J. Berthaud et al, *supra*). All natural diploids, including diploids of *T. bravum*, *T. pilosum* and *T. dactyloides* have been classified as sexual (B.L. Burson et al., Apomixis and Sexuality in Eastern Gamagrass, 30 Crop Sci. 86-89 (1990); O. Leblanc et al., Megasporogenesis and Megagametogenesis in Several Tripsacum Species (Poaceae), 82 Am. J. Bot. 57-63 (1995) (hereinafter, "Leblanc 1995c"). However, the present studies indicate that weak apomixis occurs in diploid *T. laxum* (accession 76-916; 7 of 31 appropriately staged pistils were diplosporous) and diploid *T. bravum* (accession 57-652; 11 of 30 appropriately staged pistils were diplosporous). These weakly apomictic diploids are probably of dihaploid origin, i.e. they probably arose from apomictic tetraploids through reduced egg formation followed by parthenogenesis, and percentage functionality of their diplosporous embryo sacs may be low (discussed below).

O. Leblanc et al., Chromosome Doubling in *Tripsacum*: the Production of Artificial, Sexual Tetraploid Plants, 114 Plant Breeding 226-230 (1995) (hereinafter, "Leblanc 1995a"), produced artificial tetraploid *Tripsacum* plants by chromosome doubling (colchicine method) an interspecific hybrid produced between diploid *T. laxum* (possibly weakly apomictic) and diploid *T. pilosum* (also possibly weakly apomictic). The artificial tetraploids were analyzed for sexuality, and all of them were reported to be sexual.

The Leblanc 1995a report indicates mode-of-reproduction was

determined for 10 to 25 pistils per artificial tetraploid by a sucrose clearing technique alone. Absence of more detailed cytological analyses probably led the investigators to believe that their artificial tetraploids were 100% sexual. It is important to note that the investigators at that time were convinced that apomixis was caused by a single dominant "apomixis gene" (O. Leblanc et al., Detection of the Apomictic Mode of Reproduction in Maize-*Tripsacum* Hybrids Using Maize RFLP Markers, 90 Theor. Appl. Genet. 1198-1203 (1995) (hereinafter, "Leblanc 1995b")). Hence, they did not anticipate the possibility that hybridizing two sexual diploids (devoid of the apomixis gene) followed by colchicine doubling could produce weakly apomictic tetraploids. In fact, their purpose in producing this artificial "sexual tetraploid" was to hybridize it with natural apomictic tetraploids of the same genus. The parents and progeny would then be used in simple Mendelian analyses to map the hypothetical "apomixis gene."

In 1997, the declarant obtained clonal material of one of the synthetic tetraploids analyzed by Leblanc 1995a. This plant, TS50 (tetraploid sexual 50), was transferred with other *Tripsacum* accessions (Table 3) to the USDA quarantine greenhouses in Beltsville, MD. Due to quarantine restrictions, the plants were kept in Beltsville. However, the plants were allowed to open pollinate, and immature seeds were sent to the declarant for embryo rescue. Five progeny were successfully derived from the open

pollinated TS50 inflorescences, and the paternal parent of each was identified by DNA (microsatellite) analyses. The DNA tests indicated that the fathers were weakly apomictic diploid *T. laxum* (76-916; one triploid hybrid, TS50xTLa), weakly apomictic *T. bravum* (57-652; two triploid hybrids, TS50xTBa and TS50xTBb), and strongly apomictic (determined cytologically and by progeny tests, data not shown) tetraploid *T. intermedium* (two tetraploid hybrids, TS50xTIa, TS50xTIb) (Table 7). Three of these five hybrids, TS50xTIa, TS50xTBa and TS50xTLa) have flowered and have been analyzed for apomixis along with their parents.

In contrast with the report of 100% sexuality in TS50 (Leblanc 1995a), declarant found this plant to be a moderately facultative apomict. Its parents differ in photoperiodism requirements for flowering (*T. pilosum* flowers about 30 days earlier than *T. laxum*), which is one of the three major criteria identified herein for inducing apomixis (heterozygosity for photoperiodism, onset of megasporogenesis, and onset of embryo sac formation). Unfortunately, pistils of the parents have not been available to determine differences in onset of megasporogenesis or embryo sac formation (they grow in Mesoamerica and are restricted by quarantine in the US).

Nearly 50% of appropriately staged TS50 pistils (52 of 108) were forming diplosporously, and a parthenogenic embryo (large globular stage) was observed in one of several near mature

preanthetic pistils analyzed (Fig 11). These two findings indicate that TS50 has the potential of being a fully functional facultative apomict. Nevertheless, all five of the plants that were recovered from the open pollination of TS50 were produced sexually, i.e. they formed as a product of a genetically reduced egg fertilized by a genetically reduced sperm. This result suggests that the sexual embryo sacs of TS50 are much more functional than the diplosporous ones, and this may also be the case with diploids that produce both sexual and diplosporous embryo sacs.

The hybrid TS50xTIa (TS50 x *T. intermedium* - a natural near obligate tetraploid apomict) was more apomictic than its mother but less apomictic than its father. Only 11 of 16 appropriately staged pistils (69%) in the hybrid were developing diplosporously (Fig. 11). Nevertheless, parthenogenic embryo development was found in 5 of 12 near mature preanthetic pistils (Fig. 11), which strongly suggests that TS50xTIa is a fully functional facultative apomict.

The fathers of the two triploid hybrids (TS50xTBa and TS50xTLa) were the weakly apomictic diploid *T. bravum* and *T. laxum*, respectively. Though the fathers were mostly sexual, the resulting triploid hybrids were more apomictic than their mother and more apomictic than TS50xTIa. TS50xTLa, which is a backcross derivative (*T. laxum / T. pilosum //T. laxum*), may be a near obligate apomict. Six of 6 appropriately staged pistils were developing diplosporously (Fig. 11), and though older MMC were present (all of

them were much larger than normal), no functional dyads or tetrads were observed in the small sample available from the first flowering of this plant. One degenerating tetrad was observed, which emphasizes the negative influence of triploidy on sexual fertility. A smaller sample of pistils were available from the first flowering of TS50xTBa. This plant also showed no signs of sexual development, only a few huge mature MMC (probably diplosporous) and a few immature MMC. Hence, by applying the methods of the presently claimed invention, apomictic expression of a fortuitously-produced and unrecognized weakly apomictic tetraploid *Tripsacum* hybrid (TS50) was greatly enhanced.

Antennaria. The eight *Antennaria* species used in this study were chosen because they represent divergent ecological habitats and photoperiod responses (Table 2). Analyses of these species revealed greater embryological variation than was found in the more ecologically restricted *Sorghum* sample. In *Sorghum*, all lines except 9-2 reached the dyad stage at about the same time (Fig. 4). Note that the high dMS and low or negative ESI-MS values, which were associated with diplospory in *Sorghum* hybrids (Table 5), occurred in the 9-2 containing parental pairs (because of 9-2).

In contrast with *Sorghum*, variation in timing of meiotic onset (MS) in *Antennaria* was extensive (Fig. 12). This variation, coupled with that observed for EES and TET1 (Figs. 13 and 14), has

probably permitted phylogenetically frequent evolution of diplospory in *Antennaria*. Naturally occurring diplosporous polyploid A. *rosea* appears to have evolved many times through hybridization among sexual A. *corymbosa*, A. *microphylla*, A. *pulchella* (a close relative of A. *corymbosa*) and A. *umbrinella* (R.J. Bayer, Patterns of Isozyme Variation in the *Antennaria rosea* (Asteraceae: Inuleae) Polyploid Agamic Complex, 14 Systematic Bot. 389-397 (1989); R.J. Bayer, Evolution of Polyploid Agamic Complexes with Examples from Antennaria (Asteraceae), 132 Opera Bot. 53-65 (1996)). Further evidence for the frequent origin of A. *rosea* in nature is found herein. Note that dMS and EES-MS values for the parental pair A. *corymbosa/A. umbrinella* were 35.5 and negative 5.5, respectively (calculated from Figs. 12 and 13). For A. *corymbosa/A. microphylla*, these values were 21 and 9, respectively. Based on Table 5, diplospory is expected to be frequent among natural or artificial hybrids (and more frequent among amphiploids) produced from these parental pairs (especially the former). These embryological implications collaborate the genetic implications reviewed by Bayer, *supra*, with regard to the hybrid origin of apomictic A. *rosea*.

Apomictic embryo sac formation was not observed in over 100 pistils analyzed of each diploid sexual *Antennaria* species (Table 2). However, low frequency apomixis was observed in two A. *corymbosa x A. racemosa* hybrids, which are the only *Antennaria*

hybrids of many produced that have flowered to date. The parents of these hybrids differ in their photoperiod responses. In greenhouses, A. *racemosa* flowers soon after the end of vernalization, while A. *corymbosa* flowers much later. The two weakly apomictic hybrids contained diplosporous embryo sacs and aposporous initials (early stages of aposporous embryo sac formation) in about 7% and 8% of their pistils, respectively (Fig. 15).

Only low frequency diplospory, if any, was expected in the hybrids because of the low dMS (6.7) and high EES-MS (23.3) values for the respective parental pair (calculated from Figs. 12 and 13). In contrast, the moderately high dEES value for this parental pair (13.6) suggests that signals for embryo sac formation in the ovule are staggered over a longer than normal period of time. Such signals appear to have caused one or more nucellar cells, which are only one cell layer thick in *Antennaria*, to enlarge, i.e. to become aposporous initials, in about 8% of the hybrid pistils (Fig. 15). Such signals may have also caused the three micropylar megaspores, which are normally programmed for degeneration, to persist longer than normal and to enlarge as if they were young embryo sacs (Fig. 15). This occurred in about 10% of the hybrid pistils and was much more rarely observed in pistils from the parent lines. This "persistent tetrad" anomaly is not considered apomixis, but it behaves in a manner similar to apospory in that cells other than

the surviving megaspore become gametophytized (J.G. Carman, Asynchronous Expression of Duplicate Genes in Angiosperms May Cause Apomixis, Bispory, Tetraspory, and Polyembryony, 61 Biol. J. Linnean Soc. 51-94 (1997)). The aposporous initials and persistent megaspores, which were found in a sample of about 120 pistils, did not advance beyond the single nucleus stage. In contrast, the diplosporous embryo sacs (about 7 %) became multinucleate.

6. Therefore, that using the methods of the presently claimed invention apomixis was produced, or in some cases greatly increased, in the monocotyledonous genera *Sorghum* and *Tripsacum* and in the dicotyledonous genus *Antennaria*: (a) in *Sorghum* both diplosporous and aposporous embryo sac formation were produced from completely sexual parent lines; (b) in *Tripsacum* and *Antennaria* weakly apomictic plants were produced from completely sexual parent lines; and (c) in *Tripsacum* highly apomictic lines that were clearly parthenogenic were produced from weakly apomictic lines.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Date: Jan 26, 2001

John G. Carman

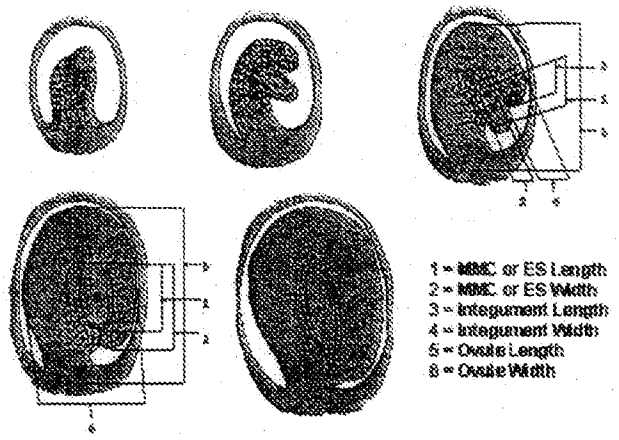

Figure 1. Ovule development stages and measurements. The figures are representative of *Antennaria* ovules, but similar measurements were made for *Tripsacum* and *Sorghum*, both of which have two integuments.

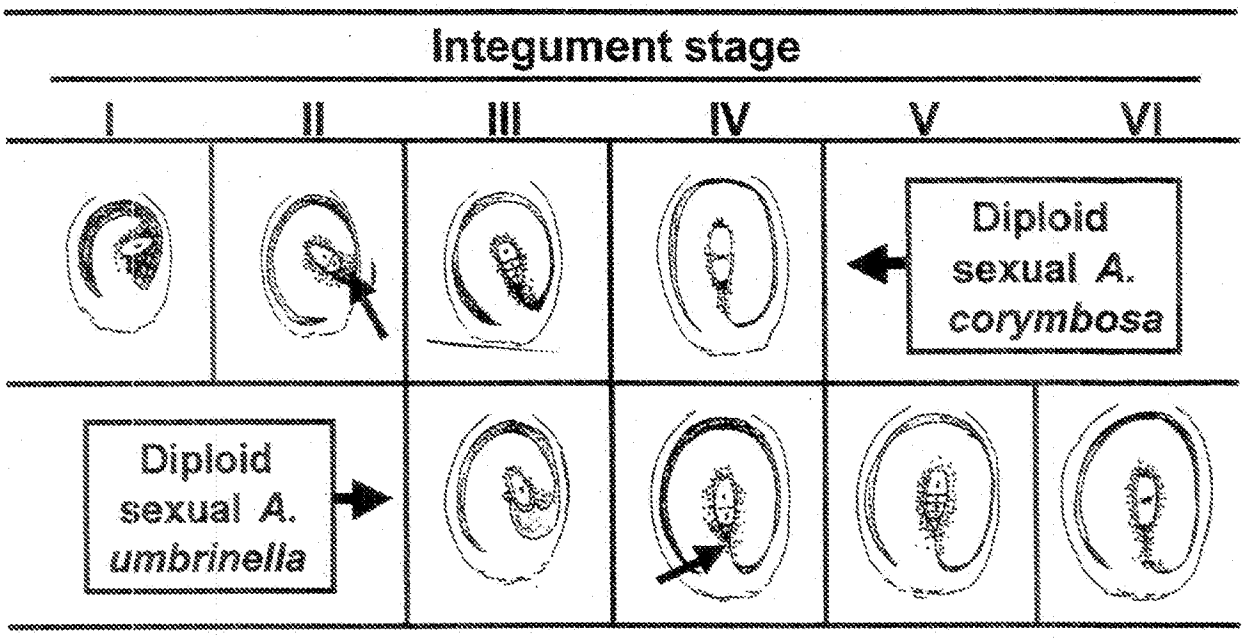

Figure 2. Simplistic representation of meiocyte, embryo sac, funiculus and integument development in two sexual progenitors of apomictic *Antennaria rosea*. Note that megagametogenesis in *A. corymbosa* begins (integument stage III) before the dyad stage is reached in *A. umbrinella* (integument stage IV).

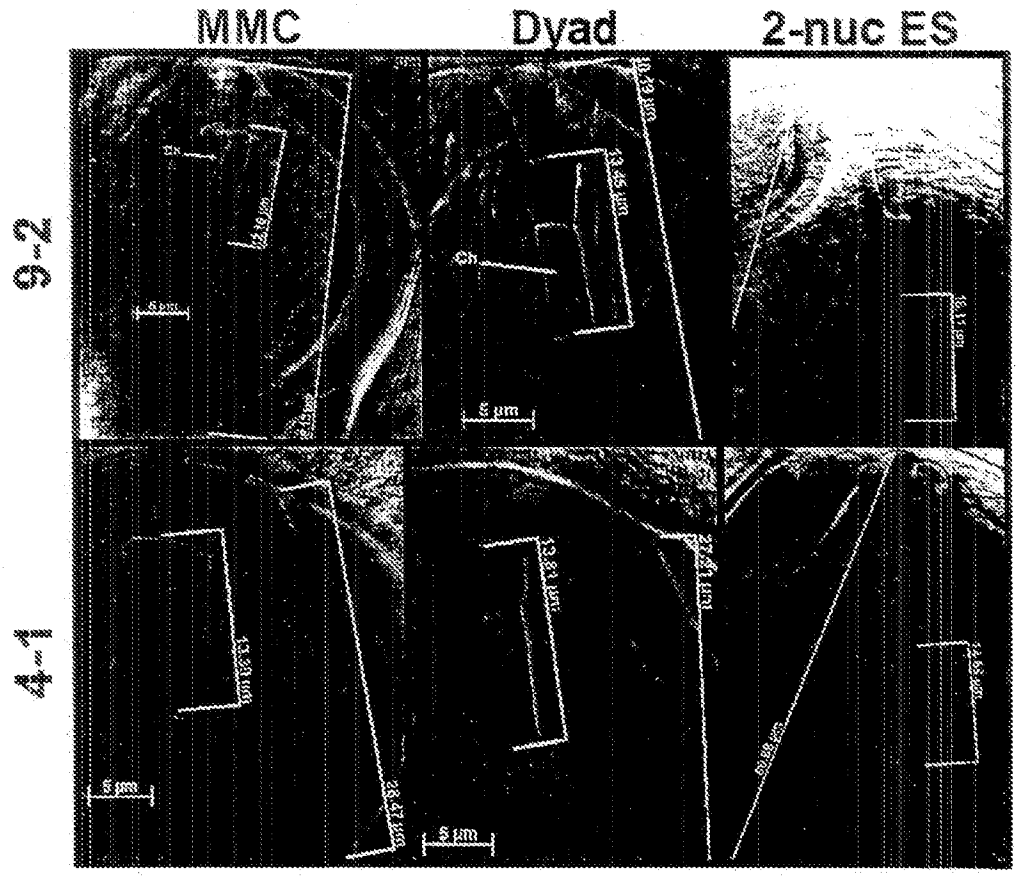

Figure 3. Ovule maturity as measured by inner integument length at the megasporocyte (MMC), dyad, and 2-nucleate embryo sac (2-nuc ES) stages for two *Sorghum bicolor* lines. Note that the dyad stage occurs earlier in 4-1 than in 9-2 (Table 1) relative to integument maturity (compare with Fig 4).

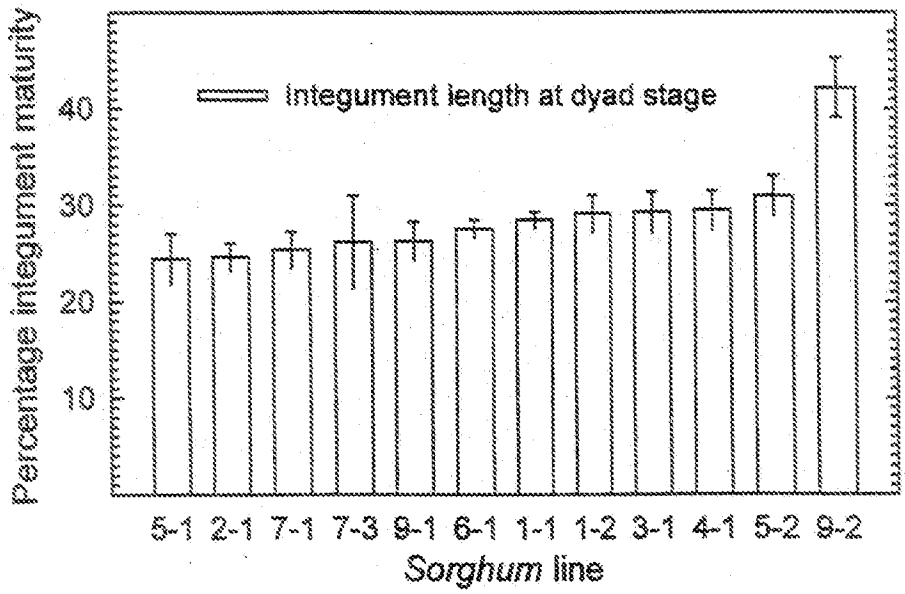

Figure 4. Percentage integument maturity (± SE) at the dyad stage of megasporogenesis (MS) for 12 *Sorghum* accessions (see Table 1 for accession identification). Percentage integument maturity of 9-2 was significantly greater ($P \leq 0.05$) than that of all other lines according to the Student-Newman-Keuls all pairwise multiple comparison procedure. Lines other than 9-2 did not differ significantly from each other.

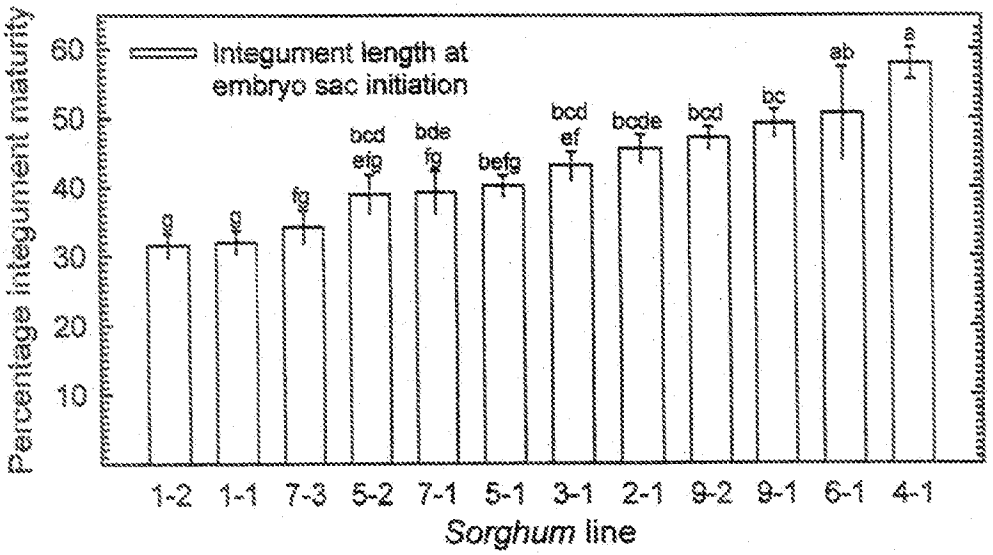

Figure 8. Percentage integument maturity (± SE) at embryo sac initiation (ESI, enlarged tetrad stage) for 12 *Sorghum* accessions (see Table 1 for accession identification). Percentage integument maturity values labeled with the same letter were not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls all pairwise multiple comparison procedure.

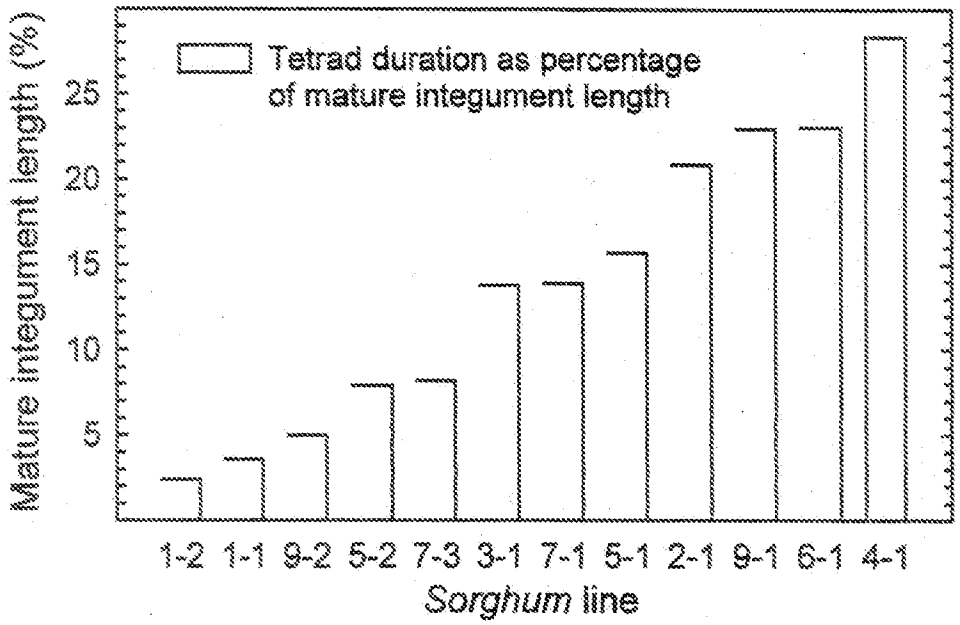

Figure 6. Integument growth during the tetrad stage (tetrad duration, TET) for 12 *Sorghum* accessions (see Table 1 for accession identification). Bars represent the difference in percentage-integument-maturity units (y axis) between the dyad stage (MS, Figure 4) and the initiation of embryo sac development (ESI, Fig 5; see Materials and Methods).

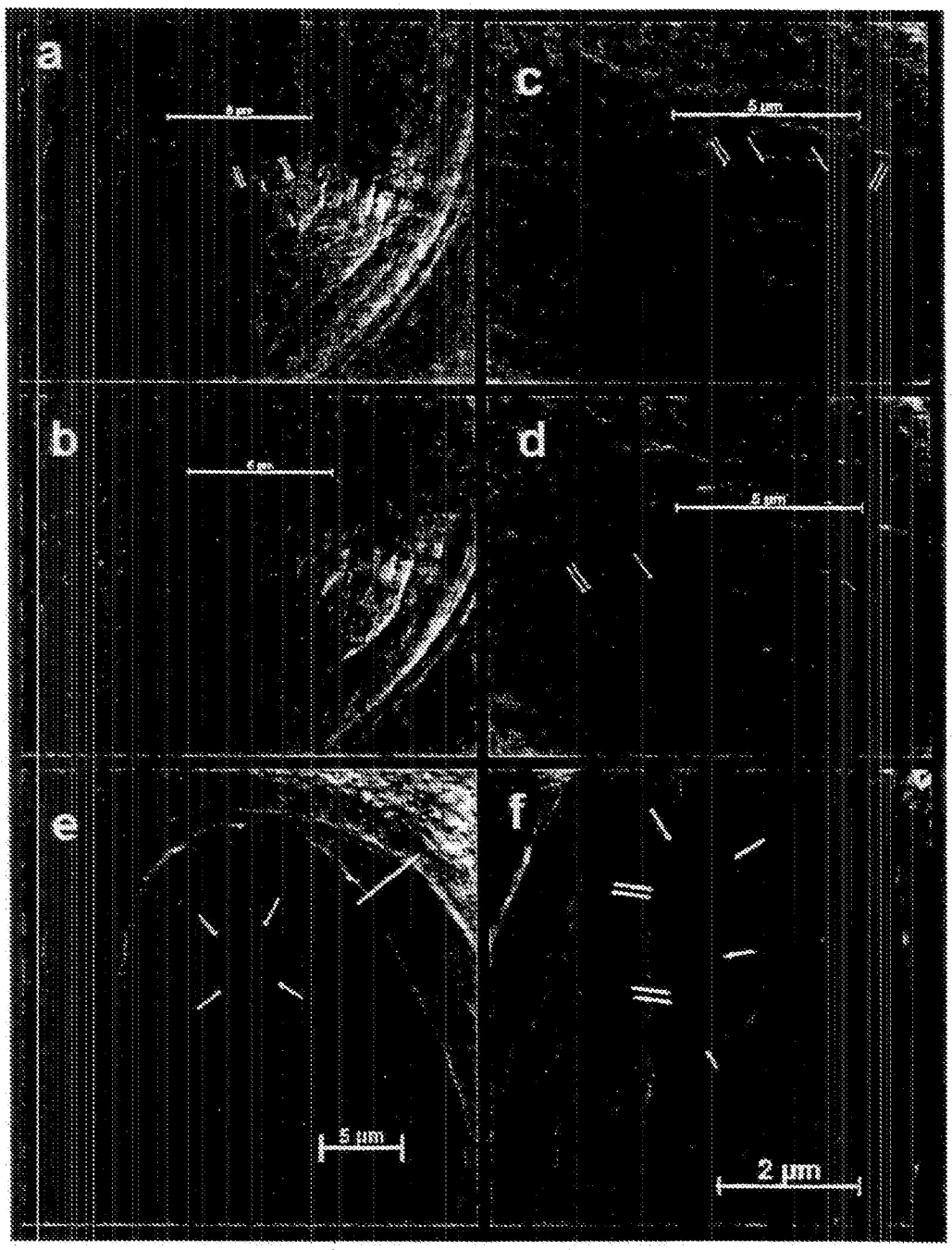

Figure 7. Aposporous (a-d) and diplosporous (e-f) embryo sac formation in *Sorghum* hybrids. (a,b) Two focal planes of the same pistil showing an early 2-nucleate aposporous embryo sac (hybrid 18, Table 4). (a) Focal plane includes micropylar half of aposporous embryo sac; double white arrows = vacuoles, single white arrows = nuclei, black arrow = degenerating micropylar megaspore. (b) Focal plane includes chalazal half of aposporous embryo sac; double black arrows = degenerating functional megaspore, single black arrows = degenerating megaspores and nucellar cells. (c,d) Two focal planes of a pistil in hybrid 84 (Table 4) showing a late 2-nucleate aposporous embryo sac and adjacent sexual 1-nucleate functional megaspore. (c) Focal plane includes aposporous embryo sac; double white arrows = vacuoles, single white arrows = nuclei, black arrow = degenerating micropylar megaspore. (d) Focal plane includes functional megaspore; double white arrows = vacuole, single white arrow = nucleus, single black arrows = degenerating megaspores. (e) Early diplosporous 1-nucleate embryo sac (hybrid 95, Table 4); white arrows = large micropylar vacuole. (f) Early diplosporous 4-nucleate embryo sac (hybrid 15, Table 4).

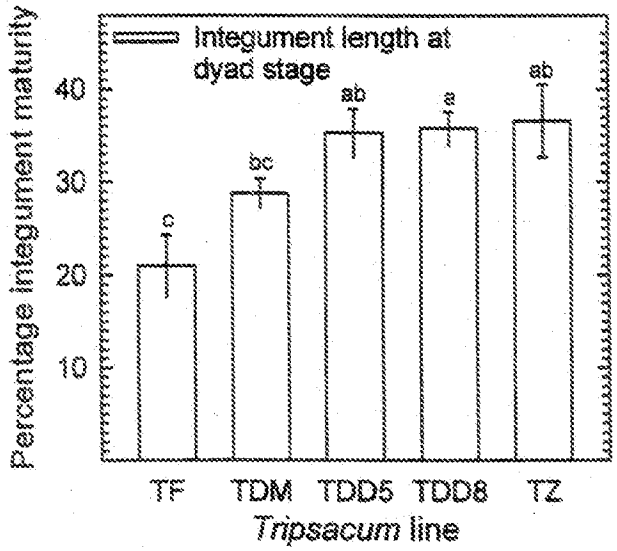

Figure 8. Percentage integument maturity (± SE) at the dyad stage of megasporogenesis (MS) for five diploid sexual *Tripsacum* accessions (Table 3). Percentage-integument-maturity values labeled with the same letter were not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls all pairwise multiple comparison procedure.

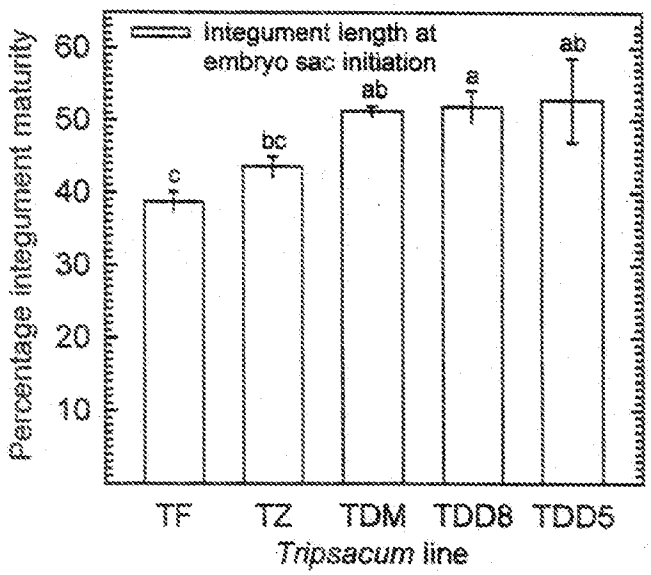

Figure 8. Percentage integument maturity (± SE) at embryo sac initiation (ESI, enlarged tetrad stage) for five diploid sexual *Tripsacum* accessions (Table 3). Percentage-integument-maturity values labeled with the same letter were not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls all pairwise multiple comparison procedure.

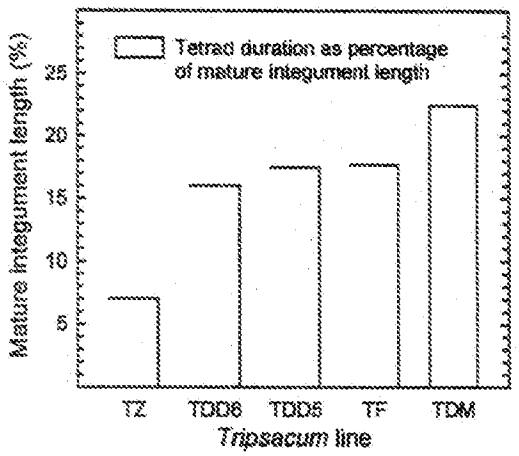

Figure 10. Integument growth during the tetrad stage (tetrad duration, TET) for five diploid sexual *Tripsacum* accessions (Table 3). Bars represent the difference in percentage-integument-maturity units (y axis) between the dyad stage (MS, Figure 7) and the initiation of embryo sac development (ESI, Fig 8; see Materials and Methods).

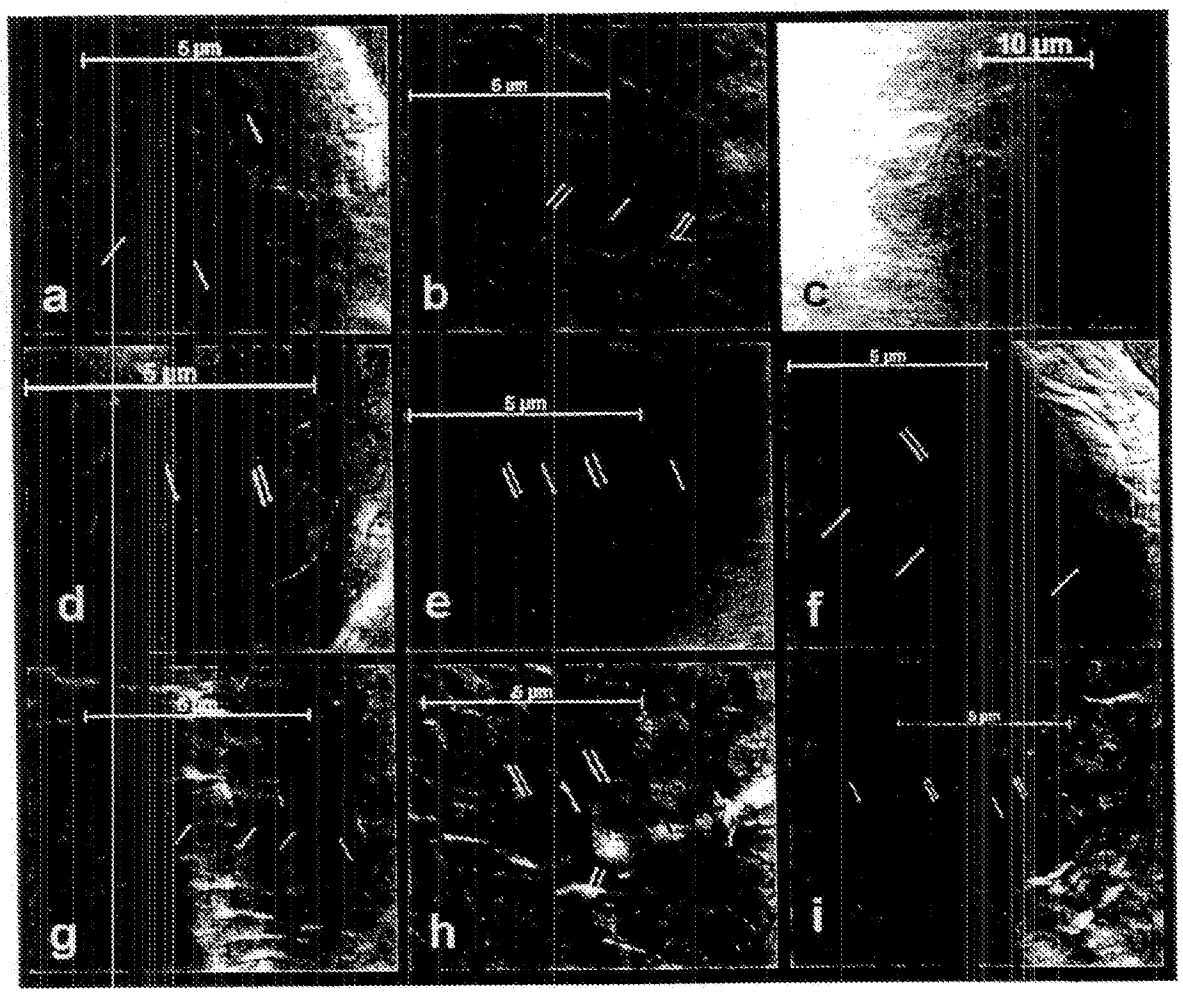

Figure11. Aposporous (a) and diplosporous (b-f) embryo sac formation in hybrids and sexual embryo sac formation in a parent line (g-1) of *Tripsacum*. (a) Degenerating tetrad and aposporous initials in TFa x TDD8b (Table 3); black arrows = degenerating megaspores, white arrows = aposporous initials. (b) Single nucleate diplosporous embryo sac in TS50 (Table 3) with two large vacuoles. (c) Parthenogenic embryo (globular stage) adjacent to unfertilized central cell (left side of embryo) in TS50. (d) 1-Nucleate diplosporous embryo sac in TFa x TDD8a. (e) 2-Nucleate diplosporous embryo sac in TDD3b x Tfal (Table 3). (f) 4-Nucleate diplosporous embryo sac in TFc x TDD8H. (g-i) Tetrad, 1-nucleate sexual embryo sac, and 2-nucleate sexual embryo sac, respectively, in TDM9c (Table 3). Note persistance of megaspore remnants (black arrows) typical in sexual 1 and 2-nucleate embryo sacs (e, f), which are not present in diplosporous 1 and 2-nucleate embryo sacs (compare with b, d, and e). In b-i, white arrows = nuclei, double white arrows = vacuoles, black arrows = degenerating megaspores and nuceliar cells.

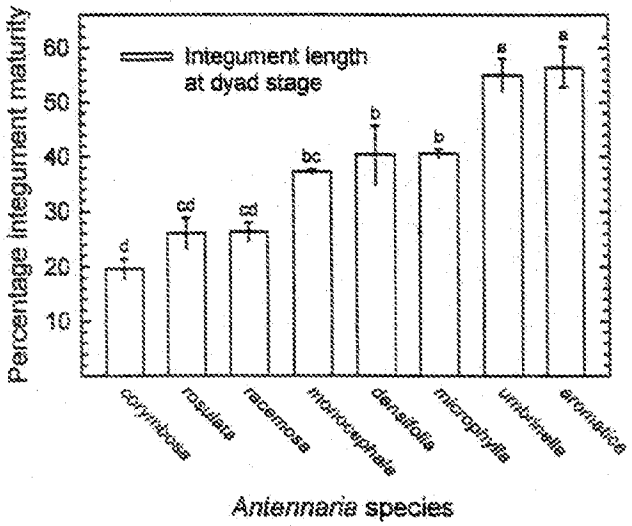

Figure 12. Percentage integument maturity (± SE) at the dyad stage of megasporogenesis (MS) for eight sexual *Antennaria* species (Table 2). Percentage-integument-maturity values labeled with the same letter were not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls all pairwise multiple comparison procedure.

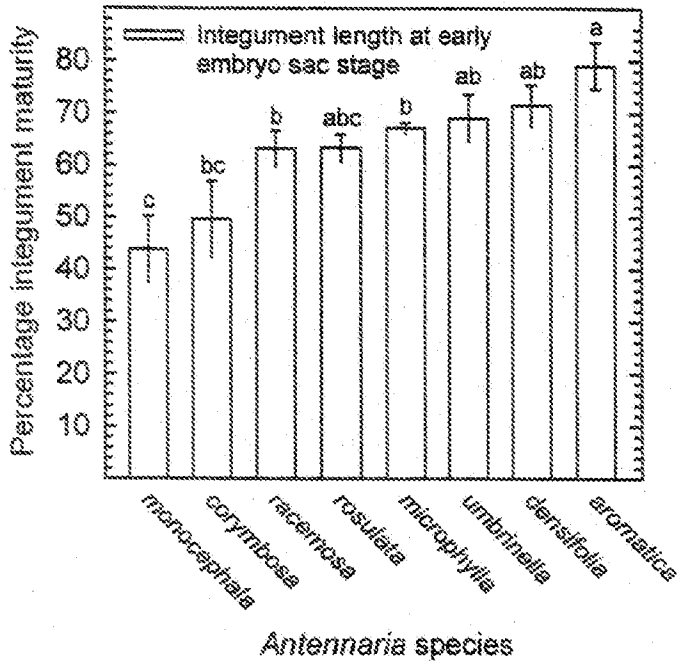

Figure 13. Percentage integument maturity (± SE) at the early embryo sac (EES, early 2-nucleate embryo sac) stage for eight sexual *Antennaria* species (Table 3). Percentage-integument-maturity values labeled with the same letter were not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls all pairwise multiple comparison procedure.

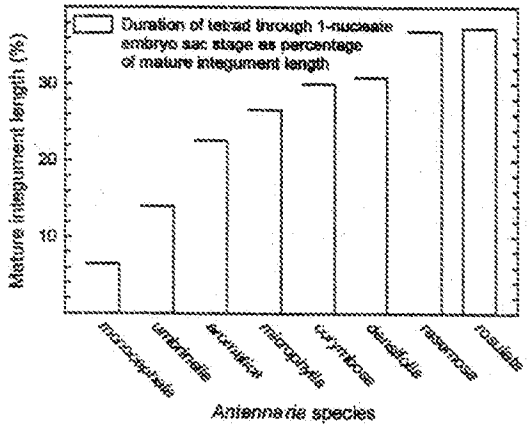

Figure 14. Integument growth during the tetrad through 1-nucleate embryo sac stage (TET1) for eight sexual *Antennaria* species (Table 2). Bars represent the difference in percentage-integument-maturity units (y axis) between the dyad stage (MS, Figure 7) and the early 2-nucleate embryo sac stage (see Materials and Methods).

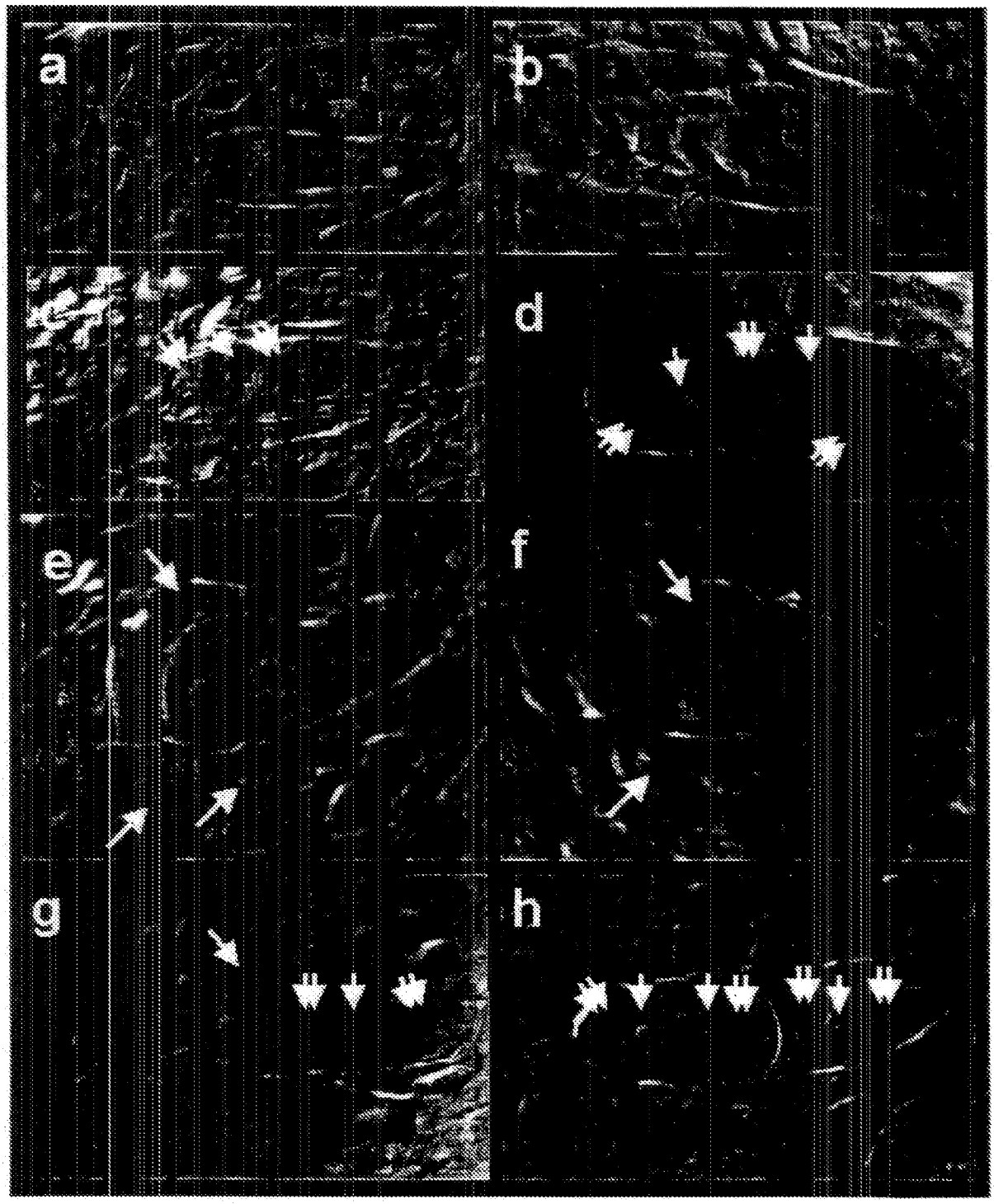

Figure 15. Sexual, apomictic and abnormal embryo sac formation in *Antennaria corymbosa* x *A. racemosa*. (a) Sexual MMC. (b) Sexual linear tetrad with normal nucellar cell development. (c) 1-Nucleate diplosporous embryo sac with vacuoles. (d) 2-Nucleate diplosporous embryo sac with vacuoles. (e) 1-Nucleate sexual embryo sac with three aposporous initials (white arrows). (f) Sexual or diplosporous MMC with two aposporous initials (white arrows). (g) 1-Nucleate sexual embryo sac with an aposporous initial (white arrow) and three persistent megaspores (see text). (h) 1-Nucleate sexual embryo sac with one degenerating and two persistent and vacuole megaspores. Compare normal nucellar degeneration in b, c, d and h with aposporous nucellar activity in e, f, and g. Unless otherwise indicated, white arrows = nuclei, double white arrows = vacuoles, black arrows = degenerating megaspores.

Table 1. Species, race, accession, country of origin and photoperiod sensitivity of *Sorghum* lines used in this study.

Line	No.	Species / race	Accession	Origin	Photoperiod sensitivity[1]
Aispuri	1-1	*bicolor* / caudatum	PI 253638	India	PS
Aispuri	1-2	*bicolor* / caudatum	PI 533817	India	PIC
IS 2942	2-1	*bicolor* / kafir	NSL 51477		PI
IS 3922	3-1	*bicolor*	NSL 51483		PI
Karad local	4-1	*bicolor* / durra	PI 533932	India	PIC
Early kalo	5-1	*bicolor*	NSL 3999		PI
Westland	5-2	*bicolor*	NSL 4003		PI
Colby	6-1	*bicolor* / durra	PI 571105	Sudan	PS
1111	7-1	*Sorghum* hybrid	PI 542649	Australia	PI
O-756	7-3	*halapense*	PI 302166	Australia	PI
Vir-5049	9-1	*bicolor* / durra	PI 562347	Sudan	PI
Agira	9-2	*bicolor* / caudatum	PI 217855	Sudan	PS

[1]PS = photoperiod sensitive; PI = photoperiod insensitive; PIC = photoperiod insensitive but converted from a photoperiod sensitive line

Table 2. Photoperiodism, days to flowering, sites collected and studied, and latitudinal range of eight *Antennaria* species evaluated in this study.

Species	Photo-perio-dism[1]	Days to flowering[2]	Sites studied	Latitudinal range (°N)
A. aromatica	LDP	Slow	6	44 – 48
A. corymbosa	LDP	Fast	6	38 – 46
A. densifolia	VLDP	Fast	3	64 – 66
A. microphylla	LDP	Slow	11	37 – 53
A. monocephala	VLDP	Fast	7	61 – 65
A. racemosa	LDP	Fast	12	43 – 52
A. rosulata	LDP	Slow	8	35 – 39
A. umbrinella	LDP	Slow	13	40 – 50

[1]LDP=long day plant, VLDP=very long day plant

[2]Slow and Fast=vernalized plants require many days and few days, respectively, to flower under VLDP conditions

Table 3. Ploidy level (36=diploid), photoperiodism (PP), and latitude of collection site for nine *Tripsacum* lines evaluated in this study.

Species	Line	Accession[1]	2n	PP[2]	Lat (°N)
T. bravum	TB	CIM 57-632	36	SDP-slow	19
T. dactyloides dactyloides	TDD3	WW 1253	36	LDP	40
T. dactyloides dactyloides	TDD8	WW 2436	36	LDP	28
T. dactyloides meridionale	TDM	MIA 34575	36	SDP-fast	6
T. floridanum	TF	MIA 34719	36	SDP-fast	28
T. intermedium (?)	TI	?	72	SDP-fast	?
T. laxum	TL	CIM 76-916	36	SDP-slow	18
T. laxum / pilosum	TL/TP	CIM TS50	72	SDP-slow	
T. zopilotense	TZ	CIM 7129-4	36	SDP-slow	17

[1]CIM=CIMMYT; WW=USDA-ARS, Woodward, Oklahoma; MIA=USDA, Miami, Florida
[2]SDP=short day plant, LDP=long day plant; slow and fast=plants require many days and few days, respectively, to flower under conducive photoperiods

Table 4. Frequency of aposporous (APO), diplosporous (DIP) and total apomictic pistil formation in hybrids produced from 12 parent lines (Table 1) divergent with respect to five floral development traits (see Materials and Methods).

			Floral divergence by floral asynchrony trait							
	Parent						Pistils	Apomictic pistils		
Hybrid	lines		P[1]	dMS[2]	ESI-MS[3]	dESI[4]	dTET[5]	obs[6]	Apo	Dip	Total (%)
15A, M	5-2	9-2	+	11.2	-3.2	8.2	-2.9	70	0	10	14.3
48, 95	3-1	9-2	+	12.9	0.9	4.1	-6.8	43	1	1	4.7
5A, D, G	5-1	4-1	+	5.1	10.7	17.7	12.7	125	6	1	5.6
16ab, H	9-1	1-2	+	2.9	2.4	-17.7	-20.6	77	5	1	7.8
76	7-3	1-2	+	3.1	2.4	-2.7	-5.8	32	1	0	3.1
84, 102	2-1	1-1	+	3.7	3.6	-13.5	-17.3	26	2	0	7.7
113	3-1	4-1	+	0.2	13.6	14.8	14.6	27	1	0	3.7
39	7-1	9-1	-	0.9	13.0	10.0	9.1	84	0	0	0
49, 59	2-1	9-1	-	1.5	19.3	3.7	2.1	58	0	0	0
78	6-1	4-1	-	1.9	21.2	7.2	5.3	36	0	1	3.0
81, 86	1-2	6-1	-	1.6	2.4	-19.1	-20.7	59	0	0	0
101	1-1	6-2	-	13.7	-10.1	15.1	1.4	79	2	0	2.5

[1]P = photoperiodism. [1]Hybrids were considered heterozygous (+) for maturity genes if one parent was PI and the other parent was PS or PIC (see Table 1)
[2]dMS = differences between parent lines in mean integument length at the dyad stage
[3]ESI-MS = difference between the smallest parental ESI value and the largest parental MS value
[4]dESI = differences between parent lines in mean integument length at the end of the tetrad stage (the beginning of megagametogenesis)
[5]dTET = difference in tetrad stage duration
[6]Pistils observed = number of cleared and observed pistils that were in the functional megaspore to 2-nucleate embryo sac stage (stages diagnostic for detecting apomixis)

Table 5. Average percentage aposporous and diplosporous embryo sac formation for hybrids classified by five floral development traits. Heterozygosity for P, dMS and ESI-MS enhances expression of diplospory, and heterozygosity for P, dESI and dTET enhances expression of apospory (note bolded characters and numbers).

	Floral divergence by floral asynchrony trait[1]					Pistils	Apomixis (%)		
Hybrids by divergence category	P	dMS	ESI-MS	dESI	dTET	obs	Apo	Dip	Total
15A, M; 48; 95	+	+	+	+/-	+/-	113	0.9	**9.7**	10.6
5A, D, G; 16ab, H; 84; 102; 113	+	+/-	+/-	+	+	255	**6.5**	0.8	6.3
76	+	-	-	-	-	32	3.1	0.0	3.1
39, 49, 59, 78, 81, 85, 101	-	+/-	+/-	+/-	+/-	322	0.6	0.3	0.9

[1]Hybrids were considered heterozygous (+) for genes controlling timing of megasporogenesis and embryo sac development (megagametogenesis) if dMS, dESI or dTET ≥ 10 or if ESI-MS < 2 (see Table 4).

Table 6. Embryological stage as a function of pistil length and *Tripsacum* species (data compiled from Leblanc and Savidan, 1994; Peel et al, 1997).

		Pistils by diploid species (%)		
Pistil length (mm)	**Pistil stage**	***T. zopilotense***	***T. dactyloides***	***T. meridionale/pilosum***
.75 - 1.00	Pre-meiotic	100	100	100
	Meiotic	0	0	0
	Embryo sac[1]	0/0	0/0	0/0
1.00 - 1.25	Pre-meiotic	34	45	33
	Meiotic	66	55	67
	Embryo sac	0/0	0/0	0/0
1.25 - 1.50	Pre-meiotic	3	16	0
	Meiotic	34	30	37
	Embryo sac	63/0	54/0	63/0
1.50 - 1.75	Pre-meiotic	0	6	0
	Meiotic	3	11	13
	Embryo sac	80/17	80/3	87/0
1.75 - 2.00	Pre-meiotic	0	0	0
	Meiotic	0	0	0
	Embryo sac	58/42	83/17	100/0
2.00 - 2.25	Pre-meiotic	0	0	0
	Meiotic	0	0	0
	Embryo sac	20/80	54/46	100/0
2.25 - 2.50	Pre-meiotic	0	0	0
	Meiotic	0	0	0
	Embryo sac	3/97	8/92	70/30

[1]mononucleate embryo sac/multinucleate embryo sac

Table 7. Alleles (BP lengths) for 18 informative microsatellite (SSR) markers by *Tripsacum* line (Table 3).

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	BNGL118	107				DUP28	77	81	98		MC1014	143			
TI		111					79	97				142			
TS50 x TIa		107	111				77	79	81	98		142	143		
TS50 x TIb		107	111				75	79	81	97		142	143		
TB		111					75	79	87	92		142			
TS50 x TBa		107	111				77	81	92	0		142			
TS50 x TBb		107	111				81	87	92	99		143	145		
TL		106	111				79					142			
TS50 x TL		107					79	81	98			142	154		
TAH		106					79					140			
TDD8		110					77								
TDD7							75					141			
TPxTZ		107					75	83	101			143	145		
TZ		107					75	77	81	98		143	145		

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	MC1017	176	194			MC1131	120	128			MC1182	84	88		
TI		164					106	121	129			74	92		
TS50 x TIa		164	176	194			120	129				74	84	88	
TS50 x TIb		176	194	208			120	129				74	84	88	
TB		176	178	0			108	133	144			78			
TS50 x TBa		176	178	194			108	120	144			78	88		
TS50 x TBb		176	194				108	117	120	128		78	88		
TL							123	125	133			88			
TS50 x TL		176	194				120	123	128			84	88		
TAH							109	111				80	96		
TDD8							111	119	123			0			
TDD7							121	125				0			
TPxTZ		182	204				120	123				86			
TZ		176					120	0				88			

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	MC1209	174				MC1325	169	177			MC1484	124	133		
TI		168	174				169	175				131	136	139	
TS50 x TIa		168	174				169	175	177			131	133	139	
TS50 x TIb		168	174				169	175	177			124	131	133	139
TB		168					173					136			
TS50 x TBa		168					169	173	177			124	136		
TS50 x TBb		168	174				169	173	177			133	136		
TL		174	180									131			
TS50 x TL		174	180				169	177				124	131		
TAH		162					129	164				136	137		
TDD8		170					175	186	187			0			
TDD7		164					167	168				136			
TPxTZ		176					169	177				124	133		
TZ		170					167	177				133			

Table 7 (cont).

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	MC1732	102	108			MC1782	261				MC1639	189	199		
TI		108	110	124	126							186	196		
TS50 x TIa		102	108	126			244	261				189	196	199	210
TS50 x TIb		108	126				244	261				186	189	199	
TB		114	118									185	0		
TS50 x TBa		102	108	118			261	265				185	199		
TS50 x TBb		102	108	118			261	265				185	199		
TL		122	130									202	210		
TS50 x TL		102	130				261					189	199	202	
TAH		98	124									185	189		
TDD8		98	133				271					184	187		
TDD7		118	128				261	269				184	187		
TPxTZ		106	116				259					201	210		
TZ		108					257	261				189	193	199	

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	MC1940	192				NC009	135				PHI021	90	98		
TI		188										92	94	98	
TS50 x TIa		192	195				119	135				90	94	98	
TS50 x TIb		188	192	200			119	135				90	92	94	96
TB		185					131					94	96		
TS50 x TBa		185	201				117	131	135			96	98		
TS50 x TBb		185	192				131	135				90	94	98	
TL							133	137				94	98		
TS50 x TL		192	195				135					94	98		
TAH		189	190									94	96		
TDD8		194	195									96	0		
TDD7		194					115	119				96	0		
TPxTZ		190					131					90	98		
TZ		190	192				115					90	0		

Line	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4	Marker	A1	A2	A3	A4
TS50	PHI031	196	212			PHI033	236				PHI065	130			
TI		189	0				213	233	239	250		126			
TS50 x TIa		189	196	212			236	239	250			126	130		
TS50 x TIb		189	196	212			213	233	236			130			
TB							226	229				127			
TS50 x TBa		196					229	236				127	130		
TS50 x TBb		196	212				226					127	132		
TL												132	153		
TS50 x TL		196	212				213	236				130			
TAH		194					240	250				140			
TDD8		190					247					139			
TDD7		180	190				251								
TPxTZ		196					236					130			
TZ		183	196				236					130			